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Antimicrobial Effect of Five Seahorse Species From Indian Coast

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Abstract: Seahorses are the good source of bioactive compounds. In the present study five species of seahorses *Hippocampus fuscus*, *Hippocampus spinnossimus*, *Hippocampus kelloggi*, *Hippocampus kuda* and *Hippocampus trimaculatus* were collected from the Gulf of Manner region of Rameshwarm. The collected seahorses were identified based on the skeletal characteristics, external morphology, and consistency features of the seahorse body surface. Antimicrobial activity of various extractions (Methanol, chloroform, and n-butanol) was investigated against 20 pathogenic strains. Among the tested 20 strains maximum activity (zone of inhibition) was recorded in *Klebsilla pneumoniae,* and *Vibrio cholerae* in the case of *H. trimaculatus* in n-butanol extracts and *Staphylococcus aureus* in the case of *H. kuda* in n-butanol extract. Some meager activity was observed in the case of *H. kellogi* towards *Streptococcus areus* and *Salmonella typhi.* Hence this present investigation suggests that seahorses will be a potential source of natural bioactive compounds.

Key words: Antimicrobial, bioactive compound, gulf of mannar, microbes, pathogens, seahorses

INTRODUCTION

Marine organisms represent an excellent source for bioactive compounds. Fishes are one of the organisms that have managed to survive in a milieu of pathogenic organisms. Today from 252 essential chemicals, which have been selected by the World Health Organization, 11.1% have plant origins, while 8.7% come from animals (Marques, 1997). Due to the aquatic environment, fish have unique anatomical and physical characteristics. Fish live in intimate contact with an environment containing both saprophytic and pathogenic microbes capable of digesting and degrading fish tissues (Ellis, 2001; Plouffe *et al.*, 2005). The slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent (Ellis, 2001) the predominant system of fish host defense. This innate immune response is essential for the survival of this whole class of animals. The defense includes many elements such as antimicrobial peptides (Cole *et al.*, 1997), and antimicrobial lipid (Ravichandran *et al.*, 2009a) polypeptides, Fernandes and Smith, (2002). Non-classical complement activation leads to release of cytokines, inflammation and phagocytosis (Ellis, 2001; Magnadottir, 2006). Concisely, fish have evolved a number of innate immune responses to defend themselves against infection.

Seahorses belong to syngnathidae of syngnathiformes in Steichthyes of vertebrate phylum. It has been widely used as a traditional medicine and invigorant for the treatment of Erectile Dysfunction (ED) due to the compellent activity like hormone from crude extract for thousands of years in the Orient. The modern pharmacological study revealed that seahorse (*Hippocampus Kuda*) has various bioactivities such as anti-tumor, anti-aging, and anti-fatigue and Ca^{2+} channel blocking (Hu *et al.*, 2000; Zhang *et al.*, 2001, 2003). However, the reports of chemical components responsible to these effects are rarely found so far. The antimicrobial effect of seahorses is rarely studied. Hence the present study is an attempt at screening of antimicrobial potence of five seahorse species collected from the Gulf of Mannar marine biosphere reserve area.

MATERIALS AND METHODS

The entire research work enlisted here is done in the Centre of Advanced Study in Marine Biology, Annamalai University from the period of December 2009-May 2010.

Collection and Identification: Five seahorse's species of *H. fuscus, H. spinnossimus, H. kelloggi, H. kuda* and *H. trimaculatus* were collected as a trash resource from Gulf of Manner region (Lat. 9º16′ N and Long. 79º8′E), southeast coast of India from December 2009 to April 2010. Seahorses were identified based on the skeletal characteristics (size and shape), external morphology. Seahorses were washed extensively with water upon collection and were transported in a solution of 100%

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methanol to the laboratory. Collected seahorses were stored at -80ºC before extraction.

Extraction: The collected seahorse species were homogenized in a homogenizer using different solvent systems. The samples were extracted with methanol, chloroform, and butanol. The organic solvents from the extracts were evaporated to dryness on a Rota vapor under vacuum at 35ºC and reconstituted with appropriate solvent before applying to the column. The water-soluble residues were lyophilized to remove all the water.

Determination of Antimicrobial activity: The spectrum of antibacterial and antifungal activity was studied by using the techniques of Bauer *et al.* (1996) against 20 pathogenic strains viz ten bacterial and ten fungal human pathogens (Table 1). Antibacterial and antifungal activity was expressed in terms of radii of Zone of inhibition and was measured in mm using a Vernier caliper scale. In brief, clinical isolates were spread plated (200 µL) onto Mueller Hinton Agar No 2 plates using a sterile spreader, taking care to distribute the microorganisms as evenly as possible over the agar surface. Extracts were prepared for the assay in the following manner. Sterile blanks (6 mm diameter) made of What man #1 filter paper, or prepunched (S and S Sterile blanks) were systematically laid out on a clean aluminum foil in such a manner that each extract was provided with three blanks per microorganism. Ten microliter of the extract were placed on the blanks by first applying 5 µL with the pipette, letting dry, then applying another 5 µL, then drying. The disc were then placed individually using a sterile forceps in appropriate grids which were marked on undersurface of the plated Petri plates and kept for incubation at room temperature for 24 h. After incubation, plates were observed for zones of inhibition that were measured with a ruler and recorded in millimeters.

Negative controls were methanol, chloroform and butanol. Positive controls are provided in ready-to-use standard discs: Tetracycline, 10 ug/disc; Flucanazole 10 ug/disc. Four discs were placed in each agar test plate (in some instances as much as 7) by using a sterile forceps and then pressing them on the agar very lightly. The plates were incubated overnight at 37ºC after which the zones of inhibition were measured. Antibacterial and antifungal activity was observed as zones of inhibition and was measured in mm using a scale.

RESULTS

Antimicrobial activity of Seahorse species: Extracts and fractions (methanol, chloroform, butanol) of seahorses *H. fuscus*, *H. spinnossimus*, *H. kelloggi*, *H. kuda* and *H. trimaculatus* were screened for antimicrobial activity against 20 pathogenic strains.

Table 1: Pathogens used for the study

Bacterial Pathogens	Fungal Pathogens
K. pneumoniae	*Aspergillus niger*
P. mirabilis	*A. flavus*
S. typhi	*A. alternaria*
E. coli	*C. albicans*
Sty. areus	*E. floccossum*
V. parahemolyticus	*T. mentagrophytes*
S. areus	*T. rubrum*
V.cholerae	*Pencillium* sp.
S. paratyphi	*Rhizopus* sp.
K. oxytoca	*Mucor* sp.

Anti bacterial screening: Out of different seahorses used for the assay the characteristic activity was brought out by *H. trimaculatus* > *H. kuda* > and *H. kellogi.* The maximum antibacterial effect was recorded against *K. pneumonia* and *V. cholerae* with 7.3 and 7.0 mm (Table 2) zone of formation shown by *H. trimaculatus* in n-Butanol extraction. In the case of *H. kuda* maximum activity is shown towards *K. oxytoca* (6.0 mm) in butanol fraction and in the case of *H. kellogi* a moderate activity towards *K. pneumoniae V. parahemolyticus* and *S. paratyphi* in butanol fraction was observed. The results were illustrated graphically in Fig. 1.

The entire screening results shows that the activity characterized so far is present in butanol fraction alone and not in other solvent systems. This indicates butanol is a good solvent system for the solubility of seahorse metabololites. A comparative antimicrobial potential of the three seahorse species were shown in Fig. 2.

Minimum inhibitory concentration: The crude *H. trimaculatus* extracts which brings the maximum antibacterial effect subjected to liquid Minimum Inhibitory Concentration (MIC) in order to assess the lethal concentration against the sensitive bacterial pathogens. This assay is done in a microtitre plate against *K. pneumoniae* and *V. cholerae* bacteria's, which shows maximum sensitization. A range of 25, 50, 60 and 80 µg/mL of concentrated sample of *H. trimaculatus* was added to the cultures of bacteria's in the micro plates and it is incubated at 27ºC for 24 h. The minimum lethal concentration was observed in 60 µg/mL in the case of *K. pneumoniae* and 80 µg/mL in *V. cholerae.*

Anti fungal screening: All the five species of seahorse extracts were subjected to the Antifungal screening. Flucanazole was used as a positive control agent. The results show that extracts are possible enough to bring antifungal effect against the fungal strains. The maximum antifungal effect was occurred in *T. mentagraphytes* with 4.0 mm zone formation and minimum of 2.0 mm in *A. flavus* strain (Fig. 3). This activity is characterized in *H. kuda* species in the methanolic and butanol fraction. Rest of the species doesn't bring any activity towards the tested strains.

Table.2. Antimicrobial activity of different crude extracts of Seahorses

Seahorses	*H.trimaculatus* (M)	*H.trimaculatus* (C)	*H.trimaculatus* (B)	*H.kuda* (M)	*H.kuda* (B)	*H.kuda* (C)	*H.kellogi* (M)	*H.kellogi* (B)	*H.kellogi* (C)
K.pneumoniae	0.1	0.1	7.5	0.1	0.1	0.1	0.1	4	0.1
P.mirabilis	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
S.typhi	0.1	0.1	0.1	0.1	4	0.1	0.1	0.1	0.1
E.coli	0.1	0.1	0.1	0.1	4	0.1	0.1	0.1	0.1
Sty.areus	0.1	0.1	0.1	0.1	6	0.1	0.1	0.1	0.1
V.parahemolyticus	0.1	0.1	4	0.1	0.1	0.1	0.1	0.1	0.1
S.areus	0.1	0.1	4	0.1	4	0.1	0.1	4	0.1
V.cholerae	0.1	0.1	7	0.1	4	0.1	0.1	0.1	0.1
S.paratyphi	0.1	0.1	0.1	5	5	0.1	0.1	4	0.1
K.oxytoca	0.1	0.1	4	0.1	6	0.1	0.1	0.1	0.1

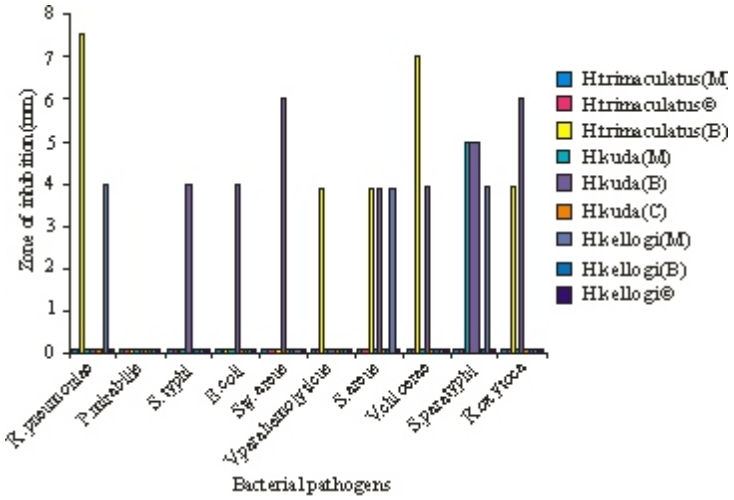

Fig.1: Antibacterial effect of seahorse species

Minimum inhibitory concentration: In the case of fungus the minimum concentration was assessed by micro dilution method and the lethal concentration of more than 80 µg/mL is required to bring the antifungal effect against *T. mentagraphytes*. The plates were incubated at 37ºC for 24 h.

DISCUSSION

In recent years, great attention has been paid to study the bioactivity of natural products and their potential pharmacological utilization. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Many of these organisms have been antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with classicical antimicrobials obtained from micro organisms (Rinehart *et al*., 1981 and Rinehart, 2000). However, majority of marine organisms are yet to be screened for discovering useful antibiotics. Seahorses are proved to possess an enormous bioactive potential like Antioxidant (Zhong *et al*., 2008) and Anti arthritis compounds (Ryu *et al*., 2008).

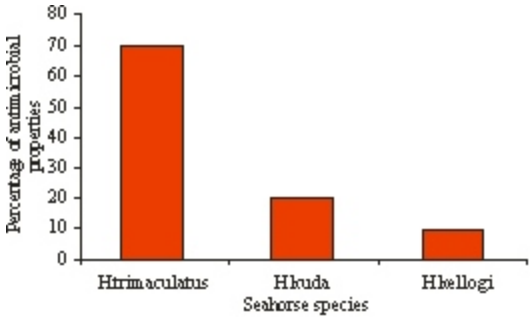

Fig.2: Comparative antimicrobial chart

Antibacterial activity have previously detected in soft corals (Kelman *et al*., 1998). The marine organisms possess an effective antibacterial properties which is observed in the case of some brachyuran crabs hemolymph which shows a vide range of antibacterial effect (Ravichandran *et al*., 2009b). The negative activity of some extracts could be attributed to the unsuitability of the method of extraction to the antibacterial screening. In the present study the maximum antibacterial effect was brought out by the *H. trimaculatus* against *K. pneumoniae* and *V. cholerae* and minimum against *V. parahemolyticus* and *Staphylococcus aereus*. However, the extraction protocol (methanol/ chloroform/ butanol) used in the

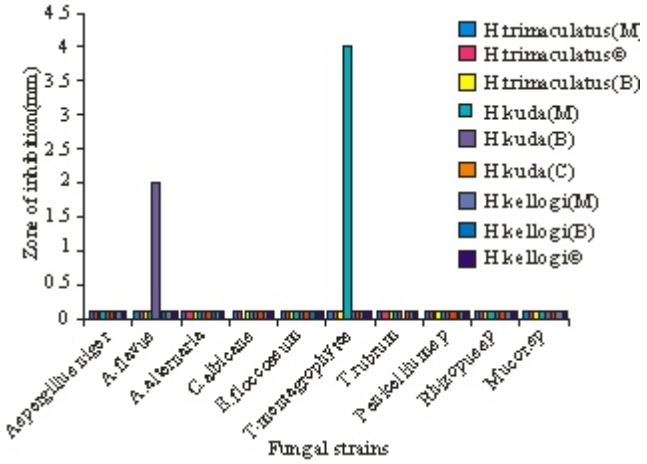

Fig.3: Antifungal effect of seahorse species

present study was selected to cover a broad range of polarities and consequently a wide range of the active substances present in the various extracts used in this study. In the present study the negative result of *H. fuscus* against *Staphylococcus aureus* and *E. coli* is highly supported by the investigations of Rafat *et al.* (2008).

Out of the five extracts studied, only one *H. kuda* has given anti trichophyton and anti *Aspergillus flavus* activity. This shows that the seahorses are extremely strange to the fungal contact and its mediated infection. There is growing evidence that pathogens have significant impacts on marine systems (Harvell *et al*., 1993). Fungal pathogens are present in reef communities and emerge when conditions favor them, and when hosts are stressed and their defenses compromised (Kim *et al*., 2000). Changing water quality can affect the host resistance to pathogens, which is an important factor in mediating host pathogen interactions (Kim, 1994). Antifungal activity in crude extracts of the soft coral Sarcophyton and the holothurian's bohadschia suggests a possible ecological function for their secondary metabolites.

The Antimicrobial property of the seahorse extracts reveals that they are high enough to bring the effect against bacterial pathogens than the fungal. It may due to the incidence of bacterial presence in their habitat induce the seahorse to produce the antimicrobial compounds. Meagre antimicrobial effect towards fungus may be of due to their rare presence in their habitat. Majority of the positive results have been recorded in the butanolic fraction and it shows that it is a good solvent system to for the solubility of bioactive compounds present in the seahorses.. Seahorses are confirmed to have a phthalate derived compounds in it to bring an effective cathepsin inhibitory activity (Li *et al*., 2008).

The future prospective of this research mission is facing towards the Analysis of possible organic compounds present in the seahorse extract and compound purification and isolation will be attempted in order to bring the exact target moiety, which is responsible for bringing the antimicrobial effect against the tested pathogenic organisms.

CONCLUSION

This research work throws a light upon the antimicrobial potence of Seahorses against a wide range of dreadful human pathogens. Seahorses are being used as a source of traditional medicine for long back in China and Thailand, this research finding adds much more essence to the biomedical potential of seahorses to fight against the microbial pathogenic diseases.

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